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Increased awareness and systematic screening for breast cancer has resulted in early detection of this disease. However, 20-30% of node negative breast carcinoma patients will develop recurrent tumors and given the highly metastatic nature of breast cancer, many of these patients progress to disseminated disease. Although developments in experimental therapies for the treatment of advanced breast cancer is promising, much of the existing treatment for advanced metastatic cancer is palliative. Among the numerous **symptoms of advanced cancer,** pain remains the most significant determinant of **quality of life.** Despite pain being the most feared symptom of advanced cancer, clinical management of cancer pain remains inadequate. Limitation in the clinical management of advanced cancer pain appears multifactorial ranging from the nature of pain itself to the irrational fear of prescribing large quantities of opiates among the treating physicians.

The goal of this investigation is to develop a novel non-opioid approach to pain management. If successful, a new therapeutic modality wil have wide spread applications in the management of terminal cancer pain and other intractable pain syndromes.

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Jay Myers 10/29/99

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5. Introduction

Increased awareness and systematic screening for breast cancer has resulted in early detection of this disease. However, 20 – 30% of node negative breast carcinoma patients will develop recurrent tumors [Weidner, 1995] and given the highly metastatic nature of breast cancer, many of these patients progress to disseminated disease [Lee, 1983]. Although developments in experimental therapies for the treatment of advanced breast cancer is promising, much of the existing treatment for advanced metastatic cancer is palliative. Among the numerous **symptoms of advanced cancer**, pain remains the most significant determinant of **quality of life** [Heim & Oci, 1993; Portenoy, 1990; Daut & Cleeland, 1982]. Despite pain being the most feared symptom of advanced cancer, clinical management of cancer pain remains inadequate. Limitation in the clinical management of advanced cancer pain appears multifactorial ranging from the nature of pain itself to the irrational fear of prescribing large quantities of opiates among the treating physicians [Fife et al, 1993; Zenz & Strumpf, 1993]. A novel non-opioid approach to pain management which requires minimal high-technology resources will have wide spread applications in the management of terminal cancer pain and other intractable pain syndromes.

In this proposal, we describe a **novel viral-vector mediated gene-therapeutic approach to pain management**. This notion is based on well documented observations that all vertebrate nervous system including humans posses an endogenous analgesic system. Of the numerous neurotransmitter systems implicated in such an endogenous analgesic system, one of the best described descending analgesic mechanism is the brain stem serotonergic input to the spinal cord. Stimulation of brain stem neurons result in release of serotonin, an amine neurotransmitter, at the spinal cord which modulates the transmission along the pain pathway. The goal of this project is to examine if enhancement of this endogenous serotonergic analgesic system can provide analgesia. We take advantage of the natural ability of virus to introduce foreign genes into non-dividing cells such as neurons. A recombinant adenovirus designed to express one subtype (5HT3) of the serotonin receptor thought to mediate part of the descending analgesic action of serotonin will be created and introduced into the subarachnoid space. The intrathecally administered recombinant adenovirus will transduce the 5HT3 receptor gene, overexpress this analgesic receptor, and thereby provide analgesia through enhancing this endogenous analgesic control.

The project utilizes molecular biological, immunohistochemical, and whole animal behavioral methods to evaluate this novel approach to pain management. The technical objectives are designed to 1) systematically evaluate the effectiveness of intrathecal recombinant adenovirus as a potential approach to providing analgesia and 2) design new viral vectors and protocols for virus co-administration with immune-modulators with the goal of overcoming the limited utility of the current generation adenovirus for actual human applications. The preliminary data accompanying this proposal indicate the high likelihood of virally mediated overexpression of spinal cord 5HT3 receptors providing analgesia. Should the proposed studies prove successful, in future studies, this adenovirus based pain management strategy will be tested in a more realistic animal model of cancer pain with the ultimate goal of bringing this approach to the clinical arena for human trials. The PI's close affiliation with the Pain Treatment Center at the University of Rochester Medical Center which is already actively involved in the management of intractable

pain in terminal cancer patients will facilitate the efficient translation of basic research to the clinics.

6. Body

Task I. To examine the dose-dependence and the time course of development of antinociceptive action of intrathecal injection of the recombinant ad(5HT3-sense).

We have experienced considerable difficulty is obtaining a consistent effect of intrathecal ad(5HT3-sense) administration on nociceptive threshold in rats. Our hypothesis is that 5HT3 receptors in the spinal cord mediates, in part, the anti-nocicpetive serotonergic input from the brain. Therefore, for expressed 5HT3 receptors to exert its desired anti-nociceptive effect, the viral vector must transduce neurons within the spinal cord. This led us to reexamine the possibility that the adenovirus may not be gaining access to the spinal cord proper after intrathecal injection.

Literature indicates that viral particles range from 20 nm (adeno associated virus) to 220 nm (herpes simplex virus). The diameter of the adenovirus is typically quoted as 90 – 120 nm. In order to investigate whether macro-particles similar in size to the viral particles gain access to the spinal cord proper, fluorescent-microspheres (Molecular Bioprobes, OR) were injected intrathecally. Twenty-four hours later, the spinal cord was harvested and the location of the microspheres visualized under fluorescence microscopy. We initially began with a 100 nm diameter microsphere and saw no entry into the spinal cord proper. Subsequent studies with even a 20 nm diameter particle demonstrated restricted distribution only in the subarachnoid space (Figure 1). There appears to be a physical barrier interposed between the spinal cord proper and the subarachnoid space limiting free diffusion of even a 20 nm diameter particle. This observation essentially precludes direct subarachoid adminstration of virus as an effective route of delivery.

Retrospective study of histological sections from animals demonstrating analgesic behavior after intrathecal ad(5HT3-sense) injection revealed presence of pial abrasion. This indicates that abrasion caused by an intrathecal catheter-induced mechanical trauma may have allowed the virus to gain access to the spinal cord proper resulting in expression of 5HT3 receptors at the correct anatomical site. Our preliminary data indicating analgesic action of ad(5HT3) most likely resulted from this technical artifact.

We have revised our approach in two ways: 1) administer ad(5HT3-sense) through direct intraspinal cord injection, and 2) develop an alternative strategy for gene-based therapy for neuropathic pain. We focused our initial efforts on the second approach. Specifically, we targeted an intracellular pronociceptive signal transduction molecule, protein kinase C-γ, strongly implicated in mediating the pain cascade [Mao et al, 1995; Malmberg et al, 1997], and began investigating the use of antisense oligonucleotide [Agrawal & Temsamani, 1996; Akhtar & Agrawal, 1997] as a potential therapeutic drug. While literature implicating PKC-γ as one mediator of nociceptive cascade abounds, no PKC-isoform specific pharmacologic antagonist exists [Hofmann, 1997]. We chose to investigate

antisense oligonucleotide as a potential gene-based PKC-γ specific antagonist. The antisense oligonucleotide approach was chosen because the PI believes that this strategy, in addition to its proven utility as a selective drug in other systems, is a technology most likely to lead into a human clinical trial in the near future [Akhtar & Agrawal, 1997; Diasio & Zhang, 1997].

Experiments performed thus far indicate that an 18mer oligonucleotide readily enters the spinal cord proper after an intrathecal injection (figure 2). Several candidate antisense oligonucleotides were identified based on sequence comparison of the classical PKCs (figure 3). Preliminary studies have identified at least one specific antisense oligonucleotide (Oligo O6) that specifically blocks PKC-γ, *in vitro* (figure 4) that exhibits antinociceptive action in rats subjected to the partial sciatic nerve ligation [Shir & Seltzer, 1990] and the formalin models of neuropathic pain (figure 5).

For this upcoming year, the following revised specific task (Task II) is proposed:

Task II:

- perform further *in vitro* antisense oligonucleotide PKC-γ knock down experiments to verify the mechanism of protein knock down.

    a. a systematic mismatch and sense control experiments to document the sensitivity of knock down.
    b. a time course experiment to develop an optimal protocol for in vivo knock down.
    c. a time course study to examine the return of PKC-γ protein level after cessation of the antisense treatment. This information will help design an *in vivo* protocol to examine whether interruption of the pain cascade will provide a long-term relief from pain. Alternatively, pain could return immediately after cessation of the antisense treatment as the cells regenerate PKC-γ.
    d. *In vivo* studies examining anti-nociceptive effects of anti-PKC-γ antisense oligonucleotide will be initiated and continued into the next year of funding.

While discouraging, we have not given up on the notion of using adenovirus as a gene-transduction vector for pain management. The intrathecal route of administration appears problematic for transduction of non-diffusible proteins such as the 5HT-3 ion channel initially proposed in this grant. The same approach may be effective for transduction of diffusible molecule such as opioid and other small peptides. While the PI does not feel further investigation of viral gene therapy as a specific task is justified at this time, exploratory work specifically targeting alternative gene-product is underway. Potential antinociceptive gene targets include peptide blockers of the NK1 and CCK receptors, both clearly indicated in mediating the neuropathic cascade.

7. Key Research Accomplishments:

- Identified a major limitation of the subarchnoid route of adenovirus administration due to anatomical barrier interposed between the subarchnoid space and the spinal cord proper.

- Developed an alternative strategy based on antisense oligonucleotide knock down of protein kinase C-γ playing a critical role in the neuropathic cascade.
- Developed methods to obtain reliable PKC-γ detection by Western blot.
- Screened antisense oligonucleotides to identify a sequence leading to selective PKC-γ protein knock down.
- Demonstrated anti-nociceptive effect of intrathecal administration of antisense oligonucleotide targeting PKC-γ.

8. Reportable Outcomes:

Manuscripts / abstracts / presentations:
Garry MG, Bezprozvannya S, Yang J "Antisense mediated downregulation of spinal NMDA receptor subunit (NR1) reduces formalin induced behaviors", **Soc. Neurosci. Abstr.,** in press.

Zollo RA, Yu J, Yang J "Antisense mediated protein kinase C-γ knockdown in vitro and in vivo", **Anesthesiol.**, in press.

Wu C, Zollo R, Garry M, Yang J "Novel targets for gene therapy in the management of pain", (in preparation).

Funding applied for based on work supported by this award:
Raymond Zollo, M.D. (PI) (Jay Yang, Mentor) "The effect of anitsense PKC-γ knockdown on neuropathic pain", Research Fellowship Award, Foundation for Anesthesia Education and Research, Rochester, MN. Awarded $59,060 (7/1/99 – 6/30/01).

9. Conclusions:

Inadequate management of cancer pain has been widely documented and may effect a reduction of quality of life in terminal patients [Heim & Oci, 1993; Portenoy, 1990; Daut & Cleeland, 1982]. Furthermore, neuropathic pain, which may be present with advanced cancer is generally resistant to opioid therapy [Payne, 1993; Arner & Myerson, 1988]. Development of a non-opiate, non-addictive, long-lasting therapy for cancer pain will revolutionarize clinical cancer pain management and alleviate patient suffering. The goal of this proposal is to investigate the feasibility of a gene-therapeutic approach to pain management. Should this approach prove feasible, in future work, this concept can be extended to overexpression of other neurotransmitter receptors implicated in the antinociceptive action (e.g. $GABA_A$, adrenergic α2, etc.) or underexpression of receptors mediating the nociceptive actions (e.g. NMDA, tachykinin, etc.) through antisense knock down.

The original goal was to use the recombinant adenovirus as a vehicle for overexpressing antinociceptive serotonin type 3A receptors in the spinal cord as a means of providing pain relief.

Our results over the last 12 months indicate that the recombinant adenovirus delivered by the subarachnoid route will not work. The physical barrier probably due to the marginal glial cells of the spinal cord prevents the adenovirus from transducing spinal cord neurons. Without efficient transduction of the spinal cord proper, the proposed approach will not work. Direct injection of the virus into the spinal cord is an alternative delivery method, but remains unsatisfactory because the approach is too invasive for easy translation to the human clinical arena.

We have taken an alternative strategy to accomplish the same goal of developing a treatment for neuropathic pain. We explored antisense oligonucleotide targeting spinal cord protein kinase C-γ as a therapeutic strategy for a non-opioid pain management. Preliminary data is promising and we hope to continue this investigation for a novel alternative to opioids for treating neuropathic pain. Antisense oligonucleotide is now well accepted as a form of therapy in humans. It is our goal to thoroughly investigate the antisense oligonucleotide targeting spinal cord PKC-γ in a preclinical model and move towards implementing a clinical trial in humans. Although the proposed revised Task for year 2 of funding is very different from the original proposal, the goal of developing a non-opioid treatment for neuropathic pain remains the same. We hope to move a step closer to the goal of developing a novel and effective pain relief with reduced side-effects for combating the diverse types of pain associated with advanced cancer. In this respect, the antisense oligonucleotide approach may be more practical than the viral vector approach.

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Zenz, M. and Willweber-Strumpf, A. (1993) *Opiophobia and cancer pain in Europe.* Lancet, **341**: 1075-6.

11. Appendices:

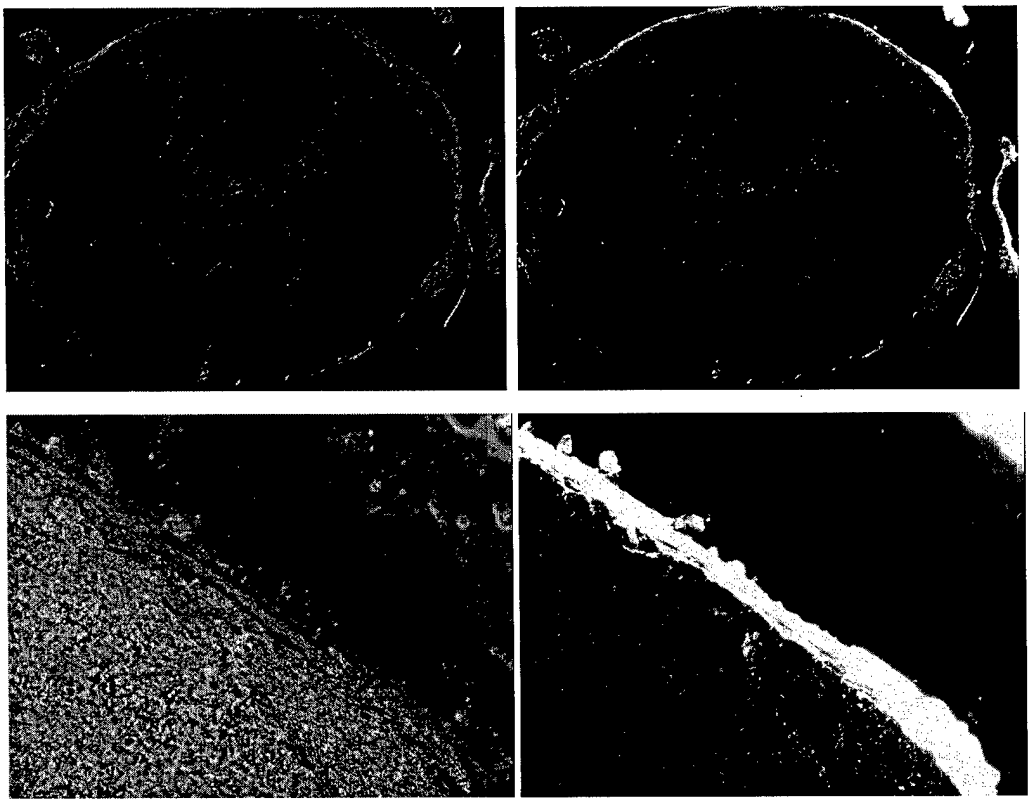


Figure 1: Microbeads (20 nm) are confined to the subarachnoid space after intrathecal injection. Phase (A,C) and fluorescent (B,D) photomicrographs of a fixed and sectioned spinal cord obtained from a rat injected with 20 nm rhodamine-tagged fluorescent microbeads 24 hours earlier. The microbeads are clearly confined in the subarachnoid space with no evidence of access into the spinal cord proper.

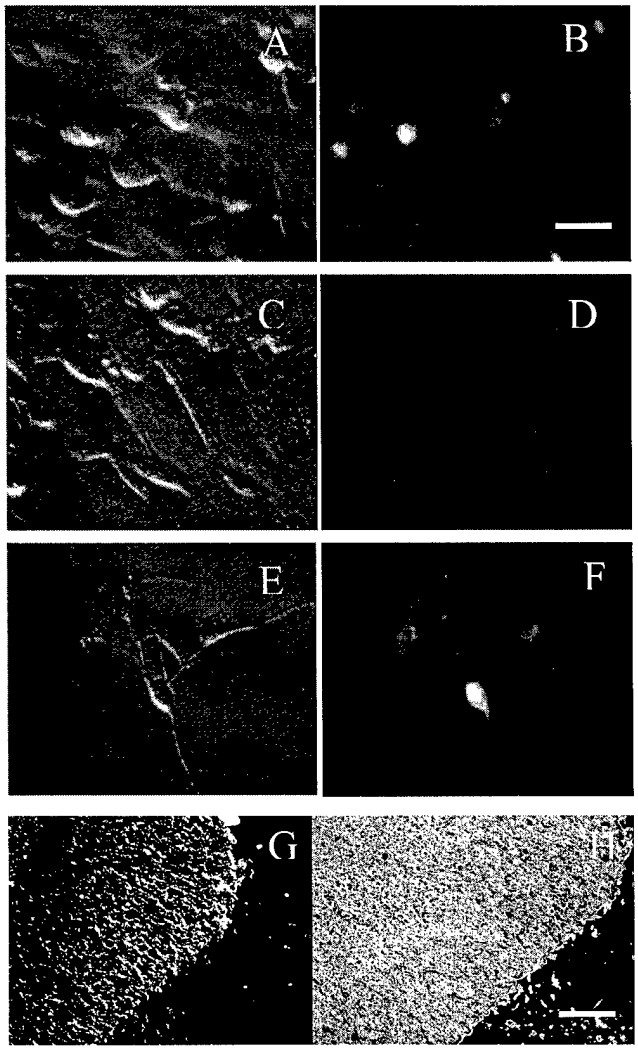
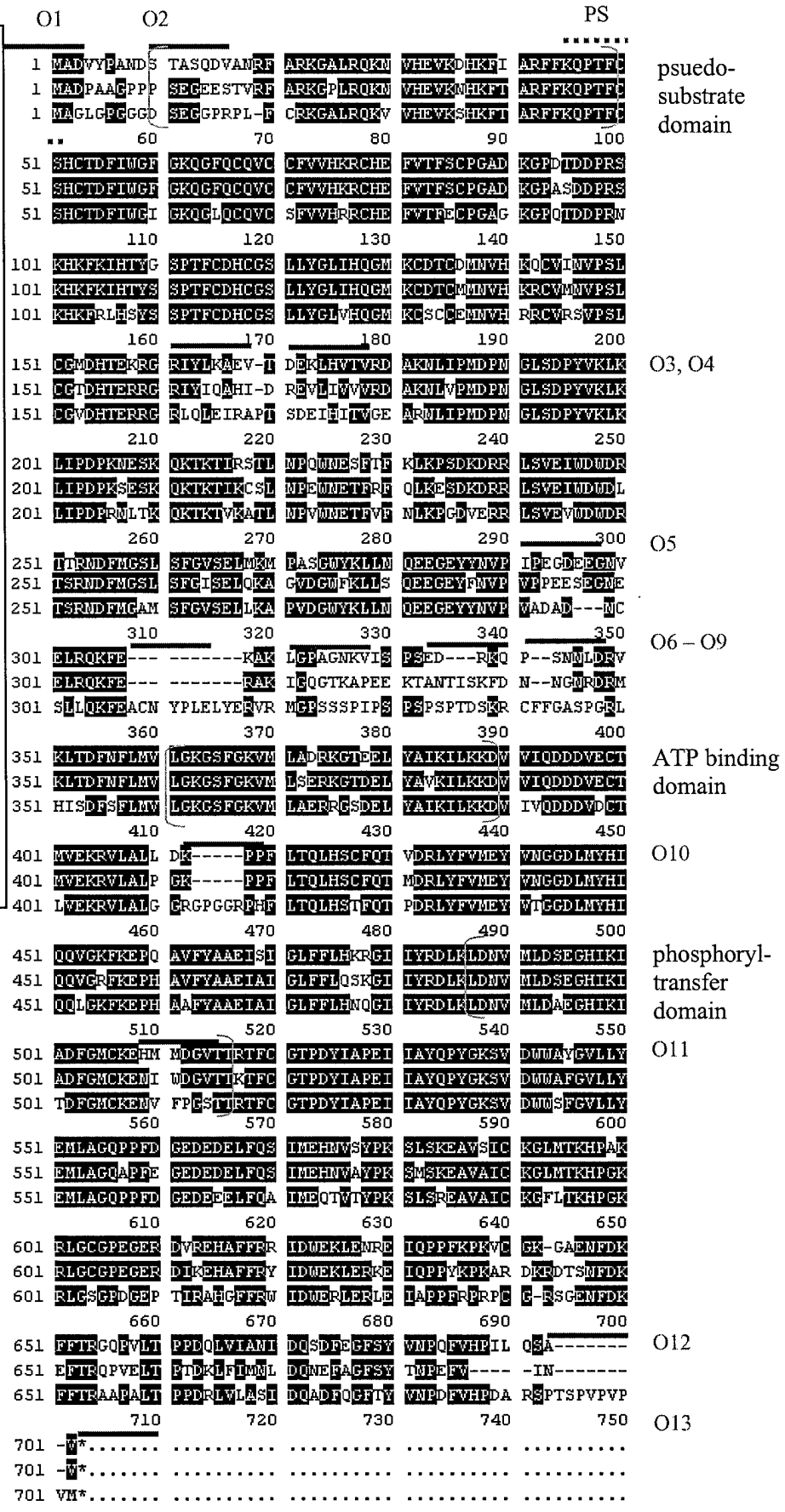


Figure 2. *Uptake of Rhodamine Labeled Oligonucleotide in various tissues.* **A**.Cultured RCGU cells treated with 5'rhodamine labeled antisense oligonucleotide (100ug/35mm plate) shown in phase contrast. Scale bar is 30 μm which applies to all panels. **B**. Same image showing rhodamine fluorescence indicating uptake of labeled antisense into RCGU cells. **C**. Untreated RCGU cells in phase contrast, and **(D)** showing absence of rhodamine fluorescence. **E**. Spinal cord neuron growing in primary culture, treated with labeled antisense oligonucleotide in phase contrast, and **(F)** showing rhodamine fluorescence. Note the intense rhodamine signal in the neuronal soma and extending into the fine neurites. **G**. Section of spinal cord from rat that received 2 intrathecal doses (100ug) of 5'rhodamine labeled antisense oligonucleotide. Spinal cord was obtained after intracardial paraformaldyhyde fixation as above. Widespread rhodamine fluorescence is seen, indicating uniform uptake of the label by the spinal cord. **H** shows the phase contrast image of the same spinal cord section. Scale bar is 150 μm which applies to G and H.

Figure 3: *Amino acid sequence alignment of classical rat PKC isoenzymes.* The entire coding sequence for PKC-α (E04372), PKC-β1 (M19007), and PKC-γ (E04371) were aligned using DNAsis (Hitachi Inc, CA). Genebank accession numbers are given in parenthesis. Regions of significant amino acid divergence were identified and thirteen oligonucleotides corresponding to the selected amino acid regions were defined as the candidate antisense oligonucleotides noted as O1 – O13 (noted in red). The PS oligonucleotide (in blue) was designed against a conserved region in the psuedosubstrate domain. Three of the well defined functional domains of the classical PKCs are identified by the green brackets. PKC-βII with 95% amino acid identity with βI was omitted from the figure for clarity.



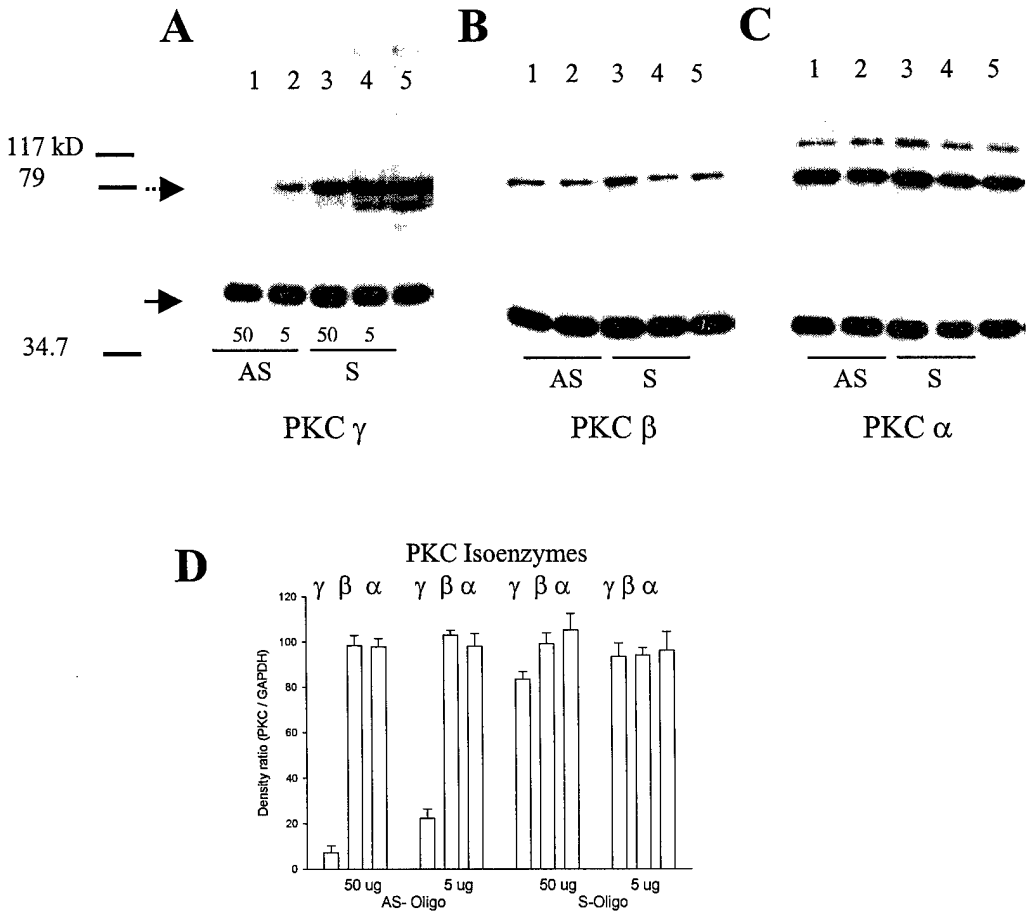


Figure 4. *Antisense oligonucleotide knockdown.* **A - C**. Western blot of C6 cells treated with 50 μg / ml (lanes 1, 3) or 5 μg / ml (lanes 2, 4) of antisense Oligo-O6 (lanes 1, 2) or sense control (lanes 3, 4) and probed with anti-PKC-γ, anti-PKC-β, or anti-PKC-α antibodies (dotted arrow). The membranes were simultaneously probed with anti-GAPDH antibody (solid arrow) to demonstrate equal loading of protein in all lanes. Lane 5 is from a water-treated control with no oligonucleotide. **D.** Densitometric analyses of 3 experiments indicate a > 95% knock down of PKC-γ protein after 48 hour treatment with 50 μg / ml. The integrated pixel values (arbitrary units) corresponding to the PKC bands were normalized to the GPADH signal (vertical axis). Approximate molecular sizes are noted on the left.

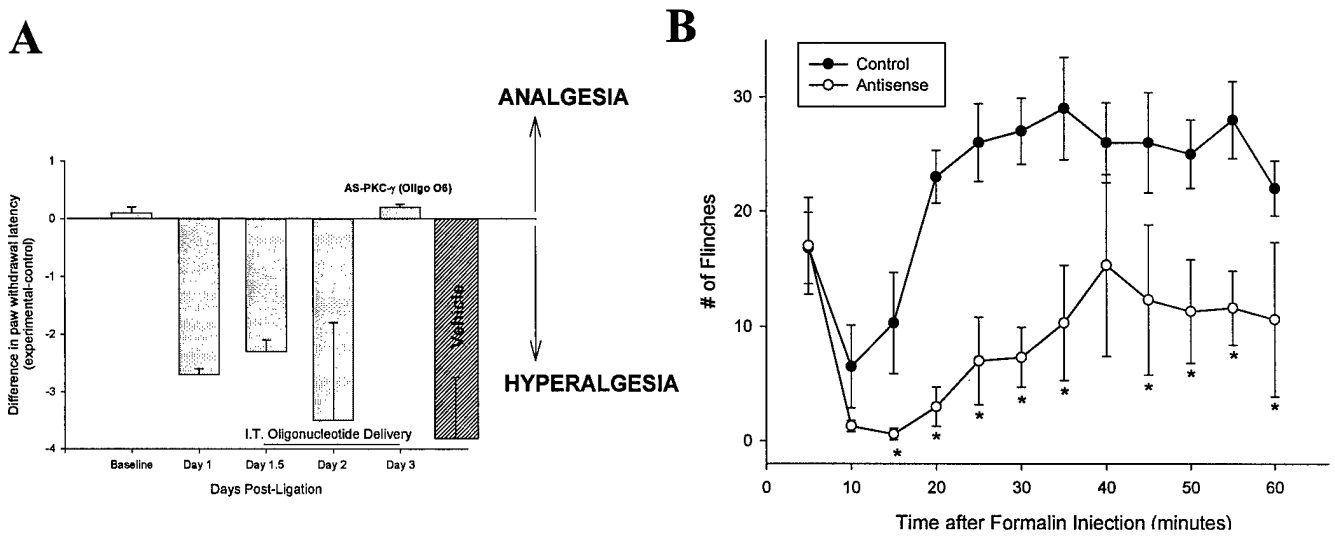


Figure 5. *A partial sciatic nerve ligation causes a sustained decrease in the paw withdrawal latency.* **A.** A partial nerve ligation experiment with (n = 4 for each group) were antisense oligo-O6 (70 μg q 12 hours x 2.5 days) were given by *i.t.* injection upon verification of a stable decrease in PWL. The indicates reversal of hyperalgesia after 2 – 2.5 days delay subsequent to initiating AS oligonucleotide treatment. Each bar is plotted as PWL in ligated limb – control limb. Thus a negative value (down ward bar) represents hyperalgesia). **B.** Behavioral response to forepaw formalin injection in control (solid) and oligo-O6 treated (open) rats. Animals (n = 5 for each group) were given five *i.t.* treatments of 70 μg AS oligonucleotide over 2 days prior to formalin injection. Phase I (0 – 10 minutes) response remained unchanged while phase II (15 – 60 minutes) response was greatly diminished. * denotes statistical significance a $p < 0.05$ by t-test.